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Supplemental Information

Rapamycin Ameliorates Age-Dependent Obesity Associated with Increased mTOR Signaling in Hypothalamic POMC Neurons

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Supplemental Information

Inventory of Supplemental Information

Supplemental Experimental Procedures follow Supplemental Data composed of six Supplemental Figures:

Figure S1 is related to Figure 1, and provides control data showing that neurons without GFP expression (indicating that they are not POMC neurons) from old POMC-GFP mice fire action potentials repeatedly (panels A and B), a growth curve showing that these mice already began to gain weight (C), and ACTH immunostaining of POMC-GFP transgenic mouse brain sections showing GFP labeling of ACTH-expressing POMC neurons (D).

Figure S2 is related to Figure 3, and shows that NPY/AgRP neurons from young mice have robust mTOR signaling, which could be suppressed by intraperitoneal rapamycin injection.

Figure S3 is related to Figure 4, and provides control showing the elimination of TSC1 protein in NPY/AgRP neurons of Agrp-cre;*Tsc1*-f/f mice.

Figure S4 is related to Figure 5, and provides control showing that Pomc-cre drives GFP expression in POMC neurons that express ACTH, and the silent POMC neurons in Pomc-cre;*Tsc1*-f/f mice can be induced to fire action potentials via current injection.

Figure S5 is related to Figure 6, and provides control that intraperitoneal rapamycin injection reduces mTOR signaling in POMC neurons of old mice.

Figure S6 is related to Figure 7, and provides control showing that intraperitoneal rapamycin injection does not silence NPY/AgRP neurons nor does it alter the biophysical properties of these neurons.

Supplemental data:

Supplemental figures

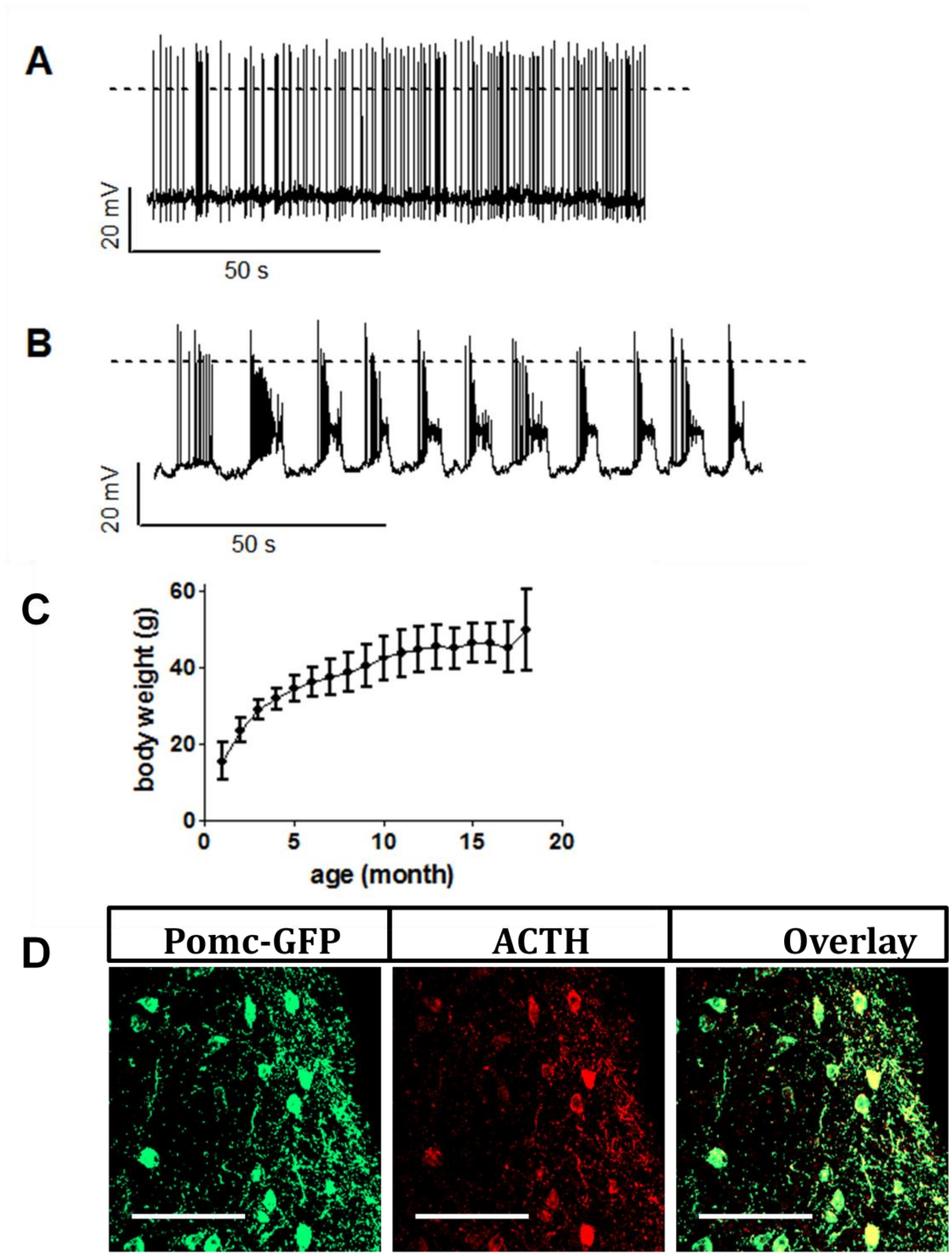

Figure S1 Representative current clamp recording of neurons without GFP label from aged (12 months old) POMC-GFP mice. Dash lines represent 0 mV. These neurons, which lacked GFP labeling for POMC neurons, from old mice were excitable and exhibited repetitive action potential firing similar to the hypothalamic arcuate neurons in young mice. Neurons without GFP labeling, which are not POMC neurons, fired action potentials repeatedly (A) or exhibited rhythmic bursting patterns of action potential firing (B), characteristic of tuberoinfundibular (TIDA) neurons in the hypothalamic arcuate nucleus (Lyons et al., 2010). Vertical scale bar is 20 mV and horizontal scale is 50 s. (C) Growth curve of male POMC-GFP mice in C57B/6 background. 5 to 49 mice were used for each time point. (D) Immunostaining with ACTH antibody of POMC-GFP transgenic mouse brain sections showing GFP labeling of POMC neurons co-expressing ACTH. Scale bars are 100 μm.

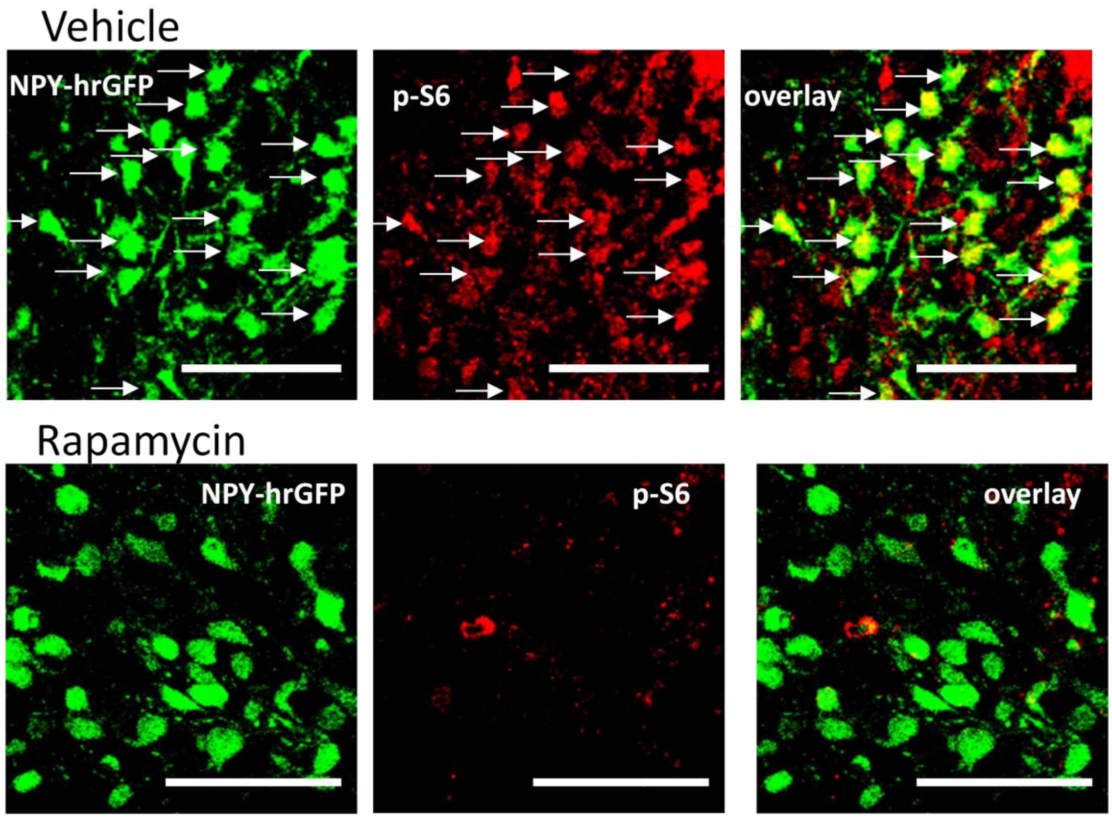

Figure S2 Representative immunostaining of the arcuate nucleus from NPY-GFP mice that had received intraperitoneal injection of either vehicle-only (top panels) or rapamycin (bottom panels) for 2 weeks. NPY/AgRP neurons were identified as GFP-positive neurons, and antibody against p-S6 was used as a marker for cells with elevated mTOR activity. Many NPY/AgRP neurons in the arcuate nucleus showed high mTOR activity; 2 weeks of rapamycin treatment suppressed most of the mTOR activity in the NPY/AgRP neurons. Scale bars are 50 μm.

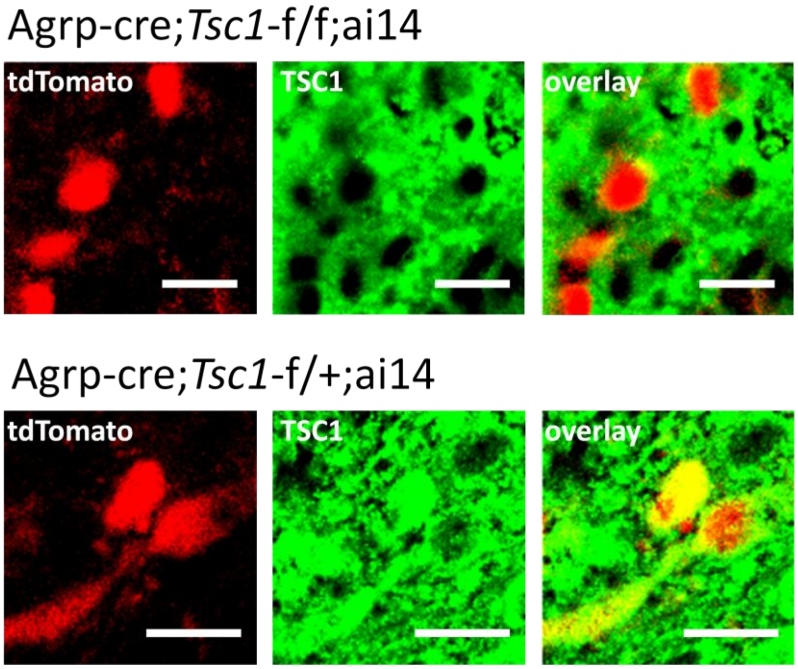

Figure S3 Representative immunostaining of the arcuate nucleus from Agrp-cre;*Tsc1*-f/f (top panels) and Agrp-cre;*Tsc1*-f/+ (bottom panels). The ai14 tdTomato line was used as a reporter to label cre-recombinase expressing neurons (red) and TSC1 was identified via a TSC1-specific antibody (green) (1:200; Epitomics Co., USA). TSC1 protein expression was absent in most of the AgRP neurons from Agrp-cre;*Tsc1*-f/f mice but it was still detectable in AgRP neurons from Agrp-cre;*Tsc1*-f/+ mice. Scale bars are 20 μm.

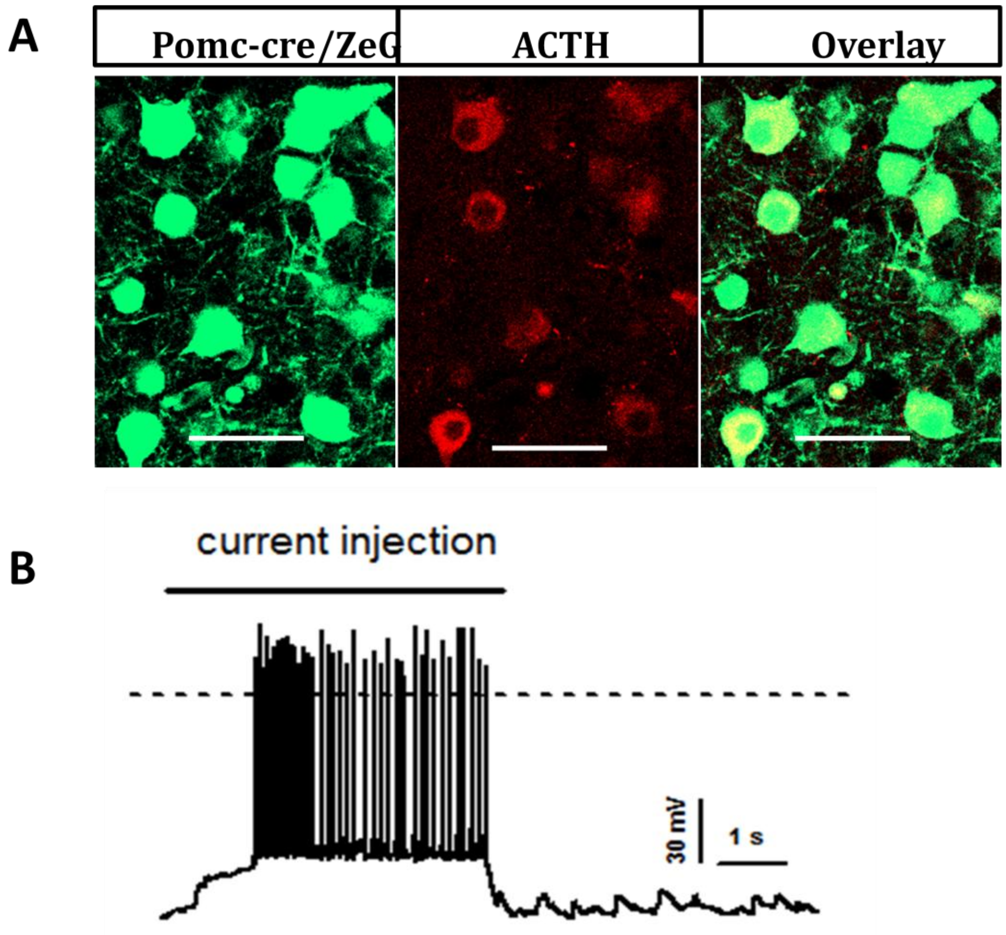

Figure S4 (A) Representative immunostaining of the arcuate nucleus from Pomc-cre;ZeG;*Tsc1*-f/f mice. ZeG line was used as a reporter to label cre-recombinase expressing neurons with GFP (green) and ACTH antibody (red) was used to confirm the efficiency and the specificity of the cre-recombinase (red) (1:200; Abcam, USA). Scale bars are 50 µm. (B) A representative current clamp recording of a POMC neuron from a Pomc-cre;ZeG;*Tsc1*-f/f mouse. Injecting 100 pA depolarized the silenced POMC neuron and triggered repetitive action potential firing.

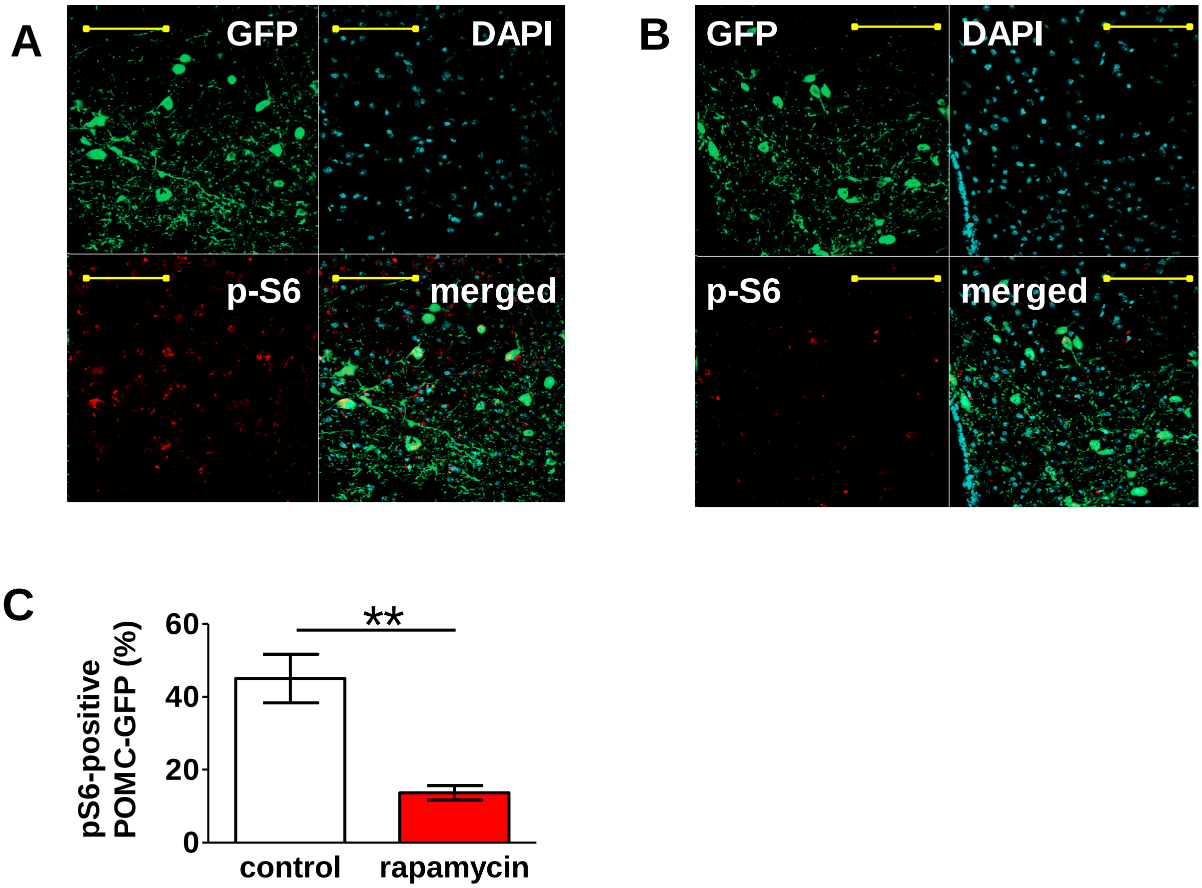

Figure S5 Intracerebral infusion of rapamycin suppressed mTOR activity in POMC-GFP neurons in aged (12 months old) mice. Representative immunostaining of the arcuate nucleus from old mice that had received either vehicle-only (A) or rapamycin (B) for 3 weeks. POMC neurons were identified as GFP-positive neurons, and antibody against p-S6 was used as a marker for cells with elevated mTOR activity. Scale bars are 100 µm. (C) Statistical analysis of p-S6 positive POMC-GFP neurons. Intracerebral rapamycin infusion significantly suppressed mTOR activity in POMC neurons from old POMC-GFP mice ($p<0.01$, Student's *t*-test; n = 3 for each age group)

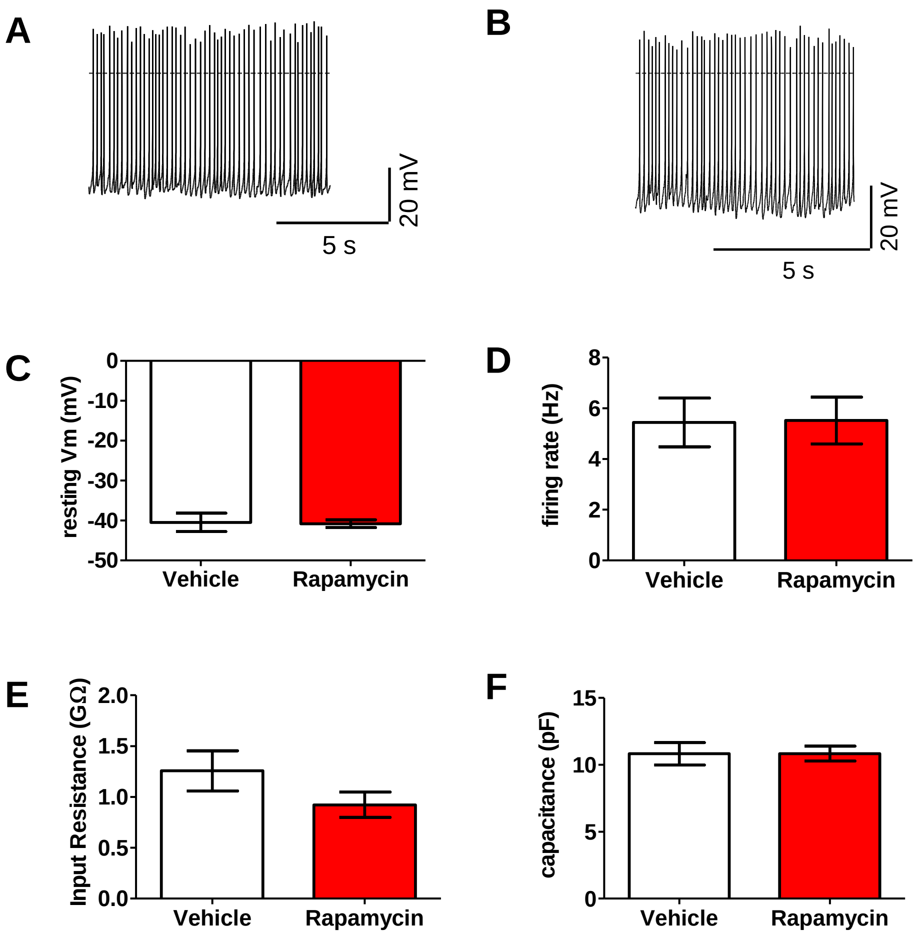

Figure S6 Biophysical properties of NPY-GFP neurons in the arcuate nucleus from NPY-GFP mice that had received either vehicle-only or rapamycin intraperitoneal injection for 2 weeks. In contrast to POMC-GFP neurons in old mice, although NPY/AgRP neurons in young mice had high levels of mTOR signaling, NPY/AgRP neurons fired repetitive action potentials from mice receiving vehicle only (A) or rapamycin injection (B). NPY/AgRP neurons from NPY-GFP mice receiving vehicle only or rapamycin injection had comparable resting membrane potentials (C), firing rates (D), input resistance (E) and capacitance (F), indicating that inhibiting mTOR does not alter the excitability in NPY/AgRP neurons ($p>0.05$, Student's *t*-test for all group comparison; $n = 7$ for vehicle-only and $n = 8$ for rapamycin group).

Supplemental Experimental Procedures

Animals. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and used protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California San Francisco. Only male mice were used for experiments and those mice for experiments were fed with chow diet and subjected to 12/12 day night cycle. At least 3 animals were used for every single experiment. POMC-GFP mice were obtained from Dr. Jeffery Friedman's lab at Rockefeller University and were backcrossed to C57B/L6 mice for more than 15 generations. Pomc-cre, *Tsc1*-flox, ZeG, NPY-GFP and ai14 mice were from Jackson Laboratory (Bar Harbor, Maine) and Agrp-cre mice were made as described previously (Xu et al., 2005).

Rapamycin (LC Laboratories) was dissolved in DMSO (Sigma) then diluted in saline for intraperitoneal injection. For intracerebral infusion, rapamycin was diluted in a solvent composed of 60% PEG400 (Sigma) and 30% cremaphor (Sigma) and the final DMSO concentration in the mixture was 10%.

Electrophysiology: 250 µm thick brain slices containing the arcuate nucleus were prepared as described previously (Sternson et al., 2005). Mice were first anesthetized by intraperitoneal Avertin injection followed by transcardiac perfusion of cold cutting solution (in mM): 110 choline chloride, 25 $NaHCO_3$, 11 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 11.6 sodium ascorbate, 3.1 sodium pyruvate, 7 $MgCl_2$, 0.5 $CaCl_2$, equilibrated with 95% O_2/5% CO_2. Brain slices were obtained by cutting in cold cutting solution using a vibratome (VT1000s, Leica). Slices were then incubated in the artificial cerebrospinal fluid (aCSF) (in mM): 126 NaCl, 21.4 $NaHCO_3$, 10 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 1.2 $MgCl_2$, 2 $CaCl_2$, equilibrated with 95% O_2/5% CO_2. An Axon200B amplifier (Molecular Devices Corp.) was used to measure membrane currents and membrane capacitance in the standard whole-cell patch-clamp configuration. Data were acquired at 5 kHz with Clampex10 software (Molecular Devices Corp.). Data with series resistances higher than 30 MΩ and holding current larger than 100 pA at -70 mV were excluded from further analysis. The intracellular solution contained (in mM): 135 Kgluconate, 15 KCl, 10 HEPES, 5 Mg_2ATP, 1 Na_3GTP, 10 sodium phosphocreatine, 0.05 EGTA and pH was adjusted to 7.2 with KOH. Pipettes were pulled from 1.5-mm borosilicate glass capillaries and had resistance of 3-5 MΩ when filled with the intracellular solution and data were included for analyses if the series resistance was lower than 30 MΩ and the holding current at -70 mV was smaller than 100 pA. All the experiments were performed at room temperature. K_{ATP} currents in POMC neurons were measured as the glibenclamide-sensitive slope conductance between -100 and -50 mV of a voltage ramp (100 ms ramp with speed of 1 V/s) as described previously (Plum et al., 2006; Speier et al., 2005). Briefly, POMC neurons were dialyzed with the same intracellular solution with low (0.5 mM) Mg_2ATP and simultaneously the slices were perfused with 300 µM diazoxide (Sigma) for ten minutes, and then 10 µM glibenclamide was added to block K_{ATP} currents.

Single cell RT-PCR: cDNA synthesis for single-cell PCR was prepared as described earlier (Liss et al., 1999; Price et al., 2008). After each recording, gentle suction was applied to the electrode and the cytoplasmic contents were collected and then aspirated into a 0.5 ml microcentrifuge tube. Prior to the reverse transcriptase reaction, the aspirated cytoplasm was treated for 30 min at 37°C with DNase. The reverse transcriptase reaction was done using an RT-PCR kit (Superscript III, Invitrogen) and the cDNA was stored at −80°C until the start of the multiplex PCR reaction. A multiplex PCR was first performed to amplify the cDNA using primers listed as below:

Kir6.2 sense, AAGAAAGGCAACTGCAACGT, antisense, CCCCATAGAATCTCGTCAGC; Kir6.1 sense, GTCTTCTAGGAGGACGCGTG, antisense, TATCATACAGGGGGCTACGC; SUR1 sense, GTGAGTTCCTGTCCAGTGCA, antisense, GTGATGTTCTCCTCCACCGT; SUR2 sense, GAGACGGAAGACATTGCCAT, antisense, CTATGATCCAGTCAGCGTGC; GFAP sense, AGAACAACCTGGCTGCGTAT, antisense, CTCACATCACCACGTCCTTG; POMC sense, GATTCTGCTACAGTCGCTCA, antisense: TCTCGGCGACATTGGGGTACA; NPY sense, GCCCAGAGCAGAGCACCC, antisense CAAGTTTCATTTCCCATCACCA.

The second reaction was a nested PCR reaction using a single set of primers to detect the presence of each gene of interest. Specific nested primer sequences are listed as below:

Kir6.2 sense,GCTGCATCTTCATGAAAACG, antisense, TTGGAGTCGATGACGTGGTA; Kir6.1 sense, GCACACAAGAACATCCGAGA, antisense, GGCTGAAAATCAGCGTCTCT; SUR1 sense, TCAAGGTTGTGAACCGCA, antisense, GGTTTCTGAGATGCGTAGGC; SUR2 sense, GGAGACCAAACTGAAATCGG antisense, TGCGTCACAAGAACAACCG; GFAP sense, AGAAAGGTTGAATCGCTGGA, antisense, CCAGGGCTAGCTTAACGTTG; POMC sense, CCCTGTTGCTGGCCCTCCTG, antisense, CTCCCCCCGCCGTCTCTTCC; NPY sense, CAGAGACCACAGCCCGCC, antisense, TCTTCAAGCCTTGTTCTGGGG.

The predicted sizes (bp) of the nested PCR-generated fragments are: 298 (Kir6.2), 448 (Kir6.1), 401 (SUR1), 215 (SUR2), 517 (GFAP), 300 (POMC) and 359 (NPY), and GFAP served as a negative quality control.

***ex vivo* α-MSH secretion assay:** Two months old mice were anesthetized with Avertin and then transcardiac perfused with cold cutting solution and the brain was cut into 1 mm thick coronal slices with a brain matrix (World Precision Instruments). Tissues containing the hypothalamic arcuate nucleus were then dissected out from the base of the brain slice and immediately transferred into aCSF and maintained at 37°C. After an initial one hour equilibration period, the hypothalamic explants were incubated for 45 min in 250 µl aCSF then transferred to solutions containing 50 nM leptin (Sigma) or 50 nM leptin plus 10 µM glibenclamide. The viability of the tissue was verified by a 45-min exposure to high potassium aCSF (KCl was increased from 2.5 mM to 50 mM, and NaCl was reduced from 126.5 mM to 78.5 mM to maintain tonicity). At the end of each period, the aCSF was frozen until it was assayed for α-MSH by a fluorescent immunoassay (Phoenix Pharmaceuticals, Burlingame, CA) according to manufacturers' instruction and all the α-MSH secretion levels from tissue explants were normalized to the α-MSH concentration in high potassium aCSF (as 100%).

Intraperitoneal glucose tolerance test (IPGTT): Glucose tolerance tests were performed after a 6-h fasting period starting from 8 am with free access to water. Each mouse received an intraperitoneal injection of glucose (2 g/kg body weight). Blood samples were obtained from the tail and the whole blood glucose level was determined using an OneTouch Ultra glucometer (LifeScan, Milpitas, CA).

Intracerebral rapamycin infusion: 12 months old male mice were acclimated in single housing condition for one week prior to the osmotic pump implantation procedure. Anesthetized mice were placed onto a stereotaxic apparatus and implanted with a brain infusion kit (Alzet) into the right lateral ventricle (anteroposterior, -0.34 mm, lateral, 1 mm and dorsoventral, -2 mm below the skull, relative to Bregma) attached to an osmotic minipump (Model1004 osmotic pump with infusion rate of 0.11 µl/hr for 4 weeks duration, Alzet). Minipumps and tubing was filled with rapamycin (10 mg/ml) or vehicle only (60% PEG400, 30% cremaphor and 10% DMSO). Using ALZAID® Chemical Compatibility Test Kit (Alzet), we confirmed that rapamycin in this PEG400/cremophor solvent was stable at 37°C up to 4 weeks, with at least 50% of rapamycin being in its active form measured by high-performance liquid chromatography. Correct pump insertion was verified by postmortem histological examination. Bodyweight and food intake were accessed daily during the whole experimental period. Mice were sacrificed at 4^{th} week for electrophysiology or immunohistochemical study.

Immunostaining: Mice were fed *ad libitum* and at 2 pm they were anesthetized by intraperitoneal Avertin injection and perfused transcardially with saline followed by 4% paraformaldehyde. Brains were removed and post-fixed overnight in 4% paraformaldehyde. Brains were then cryoprotected overnight in saline containing 30% sucrose at 4°C until they sank. Brain sections (20 µm) that were washed in blocking medium containing 0.1% Triton X-100 and 5% donkey serum (Jackson Immunoresearch Laboratories), and incubated overnight (4°C) with primary antibodies against GFP (chicken 1:200, Aves), phospho-S6 (rabbit, 1:200, Cell Signaling) and TSC1 (rabbit, 1:200, Epitomics Co.) followed by Alexa dye-tagged secondary antibodies (donkey 1:500, Invitrogen). The slides were mounted using Fluoromount G mounting medium containing DAPI (Southern Biotech) and images were acquired using a confocal microscope (Zeiss).

Supplemental References

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